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Hyperbilirubinemia Decreases Physiological Markers in Adjuvant-Induced Arthritis

K. BAUEROVA[1], F. DRAFI[1], V. KUNCIROVA[1], S. PONIST[1], D. MIHALOVA[1], P. BABAL[2], T. SYKORA[2]

[1]Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovak Republic, [2]Institute of Pathological Anatomy, FMCU and UHB, Bratislava, Slovak Republic

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Summary

There is evidence that a higher serum level of bilirubin (BIL) may be a protective factor for autoimmune diseases. We examined the effect of BIL supplementation in adjuvant-induced arthritis (AIA) where oxidative stress, inflammation and inadequate immune response are present. Male Lewis rats were randomized into groups: CO – control, AIA – untreated adjuvant-induced arthritis, AIA-BIL – adjuvant-induced arthritis administrated BIL (200 mg/kg b.w. daily i.p. during 14 days). Change of hind paw volume in the AIA-BIL group in comparison to the AIA group was significantly decreased after BIL administration. In CO and AIA groups we found almost untraceable levels of BIL. In the AIA-BIL group hyperbilirubinemia was observed. BIL administration significantly decreased plasma levels of C-reactive protein and ceruloplasmin in the AIA-BIL group in comparison to the AIA group. The values of white and red blood cells, hemoglobin and hematocrit were significantly decreased in AIA-BIL after BIL supplementation. Organs like spleen and thymus had a lower weight in AIA-BIL than in AIA. Histological findings showed decreased or even absent damage in hind paw joint of AIA-BIL animals. We observed an immunomodulatory effect of BIL on AIA development, which may also have a novel pharmacological impact.

Key words

Bilirubin • Arthritis • Immunomodulation • Inflammation markers • Blood elements

Corresponding author

K. Bauerova, Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dubravska cesta 9, Bratislava, Slovak Republic. E-mail: katarina.bauerova@savba.sk

Introduction

Bilirubin (BIL) is the yellow breakdown product of normal hem catabolism. It is excreted in bile and urine, and elevated levels may indicate certain diseases. There is evidence that higher serum level of BIL may be a protective factor for autoimmune diseases (Vitek 2005). Due to evidence of its positive effects on the organism and relatively low toxicity, we decided to study the effect of BIL on the course of adjuvant-induced arthritis (AIA), where oxidative stress, inflammation and inadequate immune response are present. This model is a good methodological tool for investigation of the pathological mechanisms occurring in rheumatoid arthritis (RA). Current research shows the potential of BIL as an immunomodulator and tissue protector in general. The aim was first to induce AIA in two groups of Lewis rats. Than we treated one AIA group with BIL to analyze the effect of BIL supplementation.

Methods

Animal protocol

Adult male Lewis rats weighing 160-180 g were obtained from the Breeding Farm Dobra Voda (Slovakia). The rats had free access to standard pellet diet and tap water. The experimental protocol was approved by the Ethics Committee of the Institute of Experimental Pharmacology and Toxicology and by the Slovak State Veterinary and Food Administration in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific

Purposes and with Slovak legislation.

Induction of adjuvant arthritis

To induce adjuvant arthritis, rats were intradermally injected with a suspension of heat-inactivated *Mycobacterium butyricum* (MB) in incomplete Freund's adjuvant (Difco Laboratories). The injection was performed near the base of the tail (Bauerova *et al.* 2011).

Experimental design and bilirubin administration

Male Lewis rats were randomized into three groups (7-8 animals in each group): healthy animals and arthritic animals not treated were referred to as CO – control group (n=7), AIA – untreated adjuvant-induced arthritis group (n=8) and AIA-BIL – adjuvant-induced arthritis group (n=8) administrated BIL (200 mg/kg of body weight daily i.p.). This dose of BIL had been described by Liu *et al.* (2008) as being effective. We used the therapeutic treatment regimen as follows: BIL was administered from day 14, when clinical signs of disease start to manifest, to day 28 of the study. Unconjugated BIL (porcine origin, AppliChem) was stored at temperature –20 °C until reconstitution. BIL was dissolved in 0.2 M sodium hydroxide to water soluble sodium salt of BIL. This solution was neutralized by 1.0 M hydrochloric acid and diluted by saline for the desired concentration of 50 mg/ml BIL. The reconstituted solution was stored at room temperature in a dark place. Handling and manipulation with BIL was under low light conditions to reduce its depletion.

Body weight of rats was measured regularly to calculate the precise application doses. On days 14, 21 and 28 blood was taken from the animals' tail. The animals were sacrificed under deep ketamin/xylasine anesthesia, blood for plasma preparation and thymus, spleen and joints were taken at the end of the experiment (day 28). Hematological evaluation and the weighting of thymus and spleen were performed immediately. All plasma samples were stored at –80 °C until biochemical analysis. Hind paw joints were fixed in 4 % formaldehyde until histological examination.

Clinical parameters evaluated: change of hind paw volume, organ weight measurement

The hind paw volume (HPV) increase was calculated as the percentage increase in the HPV on a given experimental day relative to the HPV at the beginning of the experiment. HPV was recorded on days 1, 7, 14, 21 and 28 with the use of an electronic water plethysmometer (UGO BASILE) (Bauerova *et al.* 2015). Entire organs (spleen and thymus) which were collected from each animal on day 28 were weighted on a digital scale.

Evaluation of bilirubin level in plasma

Levels of total (TB) and conjugated BIL (CB) in plasma were measured using automatic analyzer Siemens Advia 2400. The method used for evaluation was modification of the diazo method according to Garber and Jendrassik-Grof (1981). The levels were measured 24 h after the last BIL administration.

C-reactive protein

For determination of rat C-reactive protein (CRP) concentration in plasma the ELISA (Enzyme Linked Immuno Sorbent Assay) kit from Immunology Consultant Laboratories, Inc. was used. The reaction of secondary biotin-conjugated anti-rat CRP antibody was evaluated by streptavidin-HRP. The tetramethylbenzidine reaction with HRP bound to immune complex was measured at 450 nm (microplate reader Labsystems Multiskan RC). The results were calculated using the standard calibration curve on internal standards (Bauerova *et al.* 2011).

Ceruloplasmin

The enzyme activity of ceruloplasmin was measured as polyphenoloxidase at the temperature of 37 °C with the use of *p*-phenylenediamine hydrochloride as a substrate, according to Pribyl (1976).

Hematologic parameters

Blood was collected (10 µl) from the end of the tail into 80 µl of tyrode solution and 10 µl of 3.8 % citrate as anticoagulant, therefore the blood was diluted ten times. Hematologic measurements were performed by analyzer ABX Pentra 60 (Horiba Medical). The analysis is based on two major principles – electronic resistance (impedance) and light scattering.

Histological findings

The isolated hind paw joints were fixed in 4 % formaldehyde and subsequently processed by routine histological procedure, embedded in paraffin, cut in 5 µm thick slices, stained with naphtol-AS-D-chloroacetate for the detection of chloroacetylesterase activity to evaluate tissue infiltration by granulocytes.

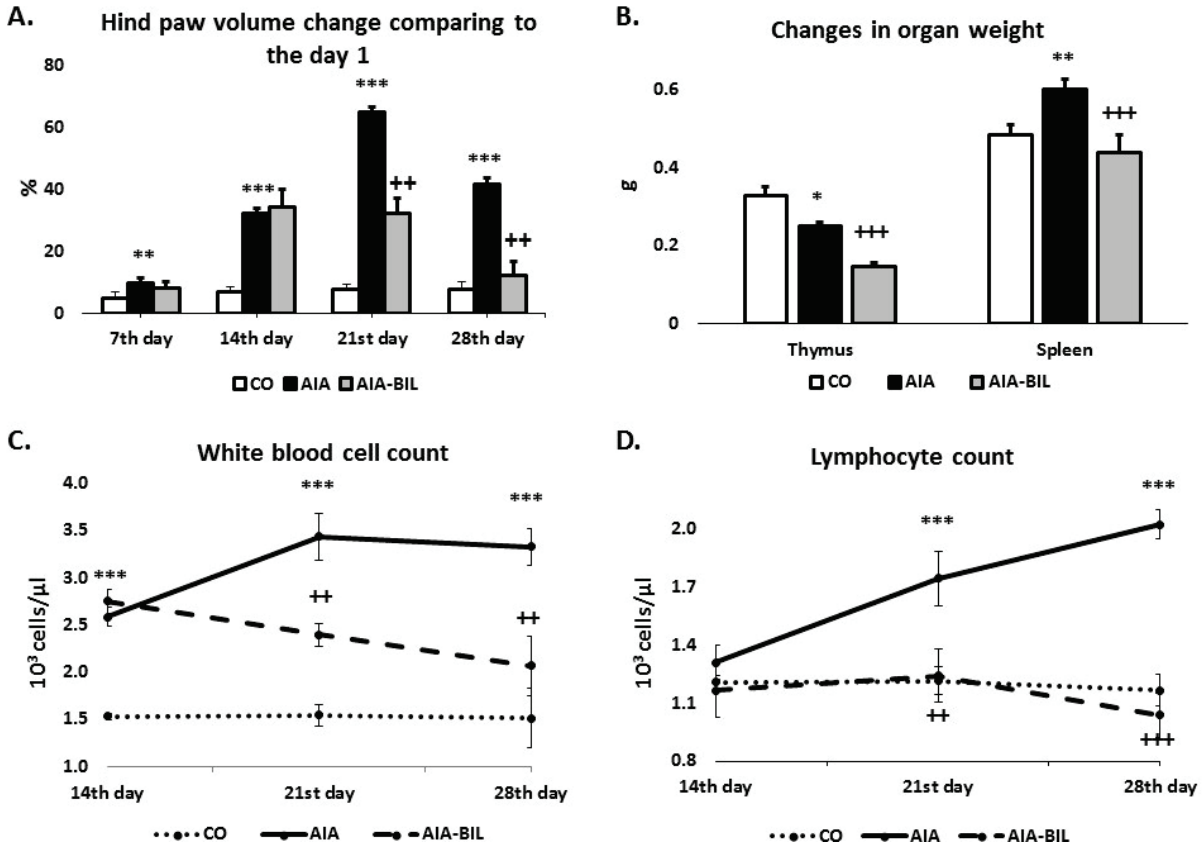

Fig. 1. A. Time profile of hind paw volume change; **B**. Change in organ weight on day 28; **C**. White blood cell count in time profile; **D**. Lymphocyte count in time profile. Data are expressed as mean ± SEM. * p<0.05 *vs* CO- control group, ** p<0.01 *vs* CO, *** p<0.001 *vs* CO, ++ p<0.01 *vs* AIA- adjuvant arthritis, +++ p<0.001 *vs* AIA.

Table 1. Plasma bilirubin levels measured as total (TB) and conjugated bilirubin (CB) on day 28. Healthy animals are described as CO, AIA is untreated adjuvant-induced arthritis group and AIA-BIL is adjuvant-induced arthritis group administrated BIL (200 mg/kg of body weight daily i.p.).

CO	**N**	
TB (µmol/l)	7	0.20±0.03
CB (µmol/l)	7	N/A
AIA	**N**	
TB (µmol/l)	7	0.29±0.04
CB (µmol/l)	7	N/A
AIA-BIL	**N**	
TB (µmol/l)	7	28.67±0.84+++
CB (µmol/l)	7	4.95±0.15

Data are expressed as mean ± SEM, 7 rats in each group. +++ statistically significant to AIA (p<0.001); N/A – undetectable levels.

Statistical analysis

The data were expressed as arithmetic mean ± SEM, with 7-8 animals in each experimental group. The untreated arthritis group was compared with healthy control animals (*) and treated arthritis groups with BIL were compared with untreated arthritic animals (+). For significance calculations, unpaired Student's t-test (two sample, unequal variance) was used with the following significance designations: extremely significant (p<0.001), highly significant (p<0.01), significant (p<0.05); not significant (p>0.05).

Results

Levels of bilirubin in plasma

The levels of TB and CB were measured on day 28. Levels of CB in CO and AIA groups are missing since these levels are very low and almost undetectable in Lewis rats (Liu *et al.* 2008). In AIA-BIL the average level of CB was 4.95 µmol/l and the average of TB level was 28.67 µmol/l, approximately one-hundred times higher than in the control animals (Table 1).

Changes of hind paw volume and organ weight. White blood cells and lymphocyte count in blood screen

Change of hind paw volume is a marker of clinical progression of the disease as it indicates edemas

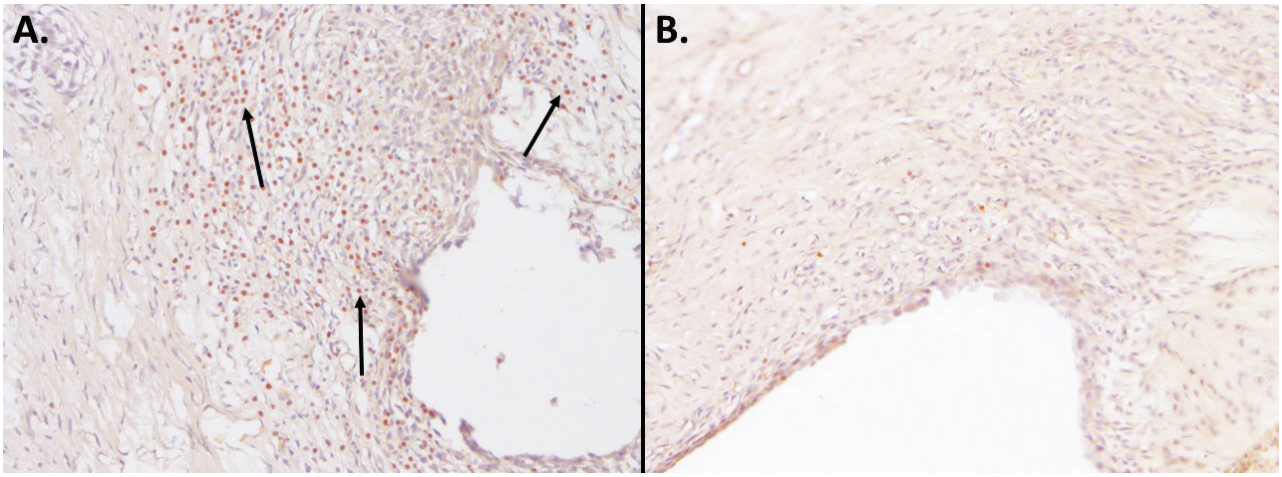

Fig. 2. Knee joints of animals with induced adjuvant arthritis. **A**. Dense granulocytic (red color) infiltrate of the inflamed joint synovia (arrows); **B**. Adjuvant arthritis treated by bilirubin administration results in reduction of inflammatory cell infiltration. Naphtol-AS-D-chloroacetate staining (magnification 100x).

of the affected joints. The results shown in Figure 1A clearly indicate that this clinical sign of AIA diminished after administration of unconjugated BIL. We observed that the change in the size of the edemas resulted also in higher activity and mobility of the treated animals. Changes in edemas of the hind paws were visible on day 7, a week after application of Freud's adjuvant. Administration of BIL started on day 14. Until day 14, the AIA and AIA-BIL groups had equal HPV. After this break point, a significant difference between the groups AIA and AIA-BIL occurred (++ $p<0.01$) when the average values of the treated animals were approx. 50 % lower than in the untreated group. This trend continued to the end of the experiment where it could be clearly seen that values of the AIA-BIL group almost dropped to the values of the CO group, exhibiting significant difference with the AIA group (++ $p<0.01$).

Spleen and thymus weights were significantly reduced in the AIA-BIL group in comparison to the AIA group (+++ $p<0.001$, Fig. 1B). Compared to CO, the AIA spleen was significantly enlarged and the thymus was significantly reduced (* $p<0.05$ – thymus, ** $p<0.01$ – spleen, Fig. 1B).

As evident from Figure 1C, 14 days after induction of AIA the inflammation is already in progress. White blood cells (WBC) in AIA and AIA-BIL groups are equal and both are significantly higher in comparison with the CO group (*** $p<0.001$, Fig. 1C). Significant changes of WBC in AIA-BIL compared to AIA are visible on day 21 (++ $p<0.01$, Fig. 1C) and this trend is maintained to the end of the experiment. However, leukocytes of the AIA group remained significantly high in comparison to CO (*** $p<0.001$, Fig. 1C). On the other hand, lymphocyte count on day 14 was equal in all three groups. On day 21 we observed a rise of lymphocytes in AIA, with the increase remaining till day 28 (*** $p<0.001$, Fig. 1D). Compared to AIA, lymphocytes in AIA-BIL were significantly lower – copying levels in CO (+++ $p<0.001$, Fig. 1D).

Histological findings

Histological findings proved almost no inflammatory infiltration of granulocytes in the hind paw joints of animals treated with BIL (Fig. 2B) compared to AIA (Fig. 2A).

Changes of inflammatory markers – CRP and ceruloplasmin

The level of CRP, an important laboratory marker of inflammation, was significantly reduced on day 28 in the AIA-BIL group in comparison with AIA (Table 2, ++ $p<0.01$), being practically equal to CO. The levels in the AIA untreated group were significantly high in comparison to the CO group (Table 2, ** $p<0.01$).

Ceruloplasmin is regarded a non-specific acute phase reactant in serum and its level of change correlates with the pattern of changes in CRP. Values on day 28 indicate that unconjugated BIL administration reduced also this inflammatory marker significantly in the AIA-BIL group compared to AIA (++ $p<0.01$, Table 2).

Hematological changes

On all experimental days monitored, red blood cells (RBC), hemoglobin (HGB) and hematocrit (HCT)

were evaluated and no significant change in these parameters in AIA compared with CO was observed (Table 3).

Table 2. Plasma levels of CRP and ceruloplasmin measured on day 28. Healthy animals are described as CO, AIA is untreated adjuvant-induced arthritis group and AIA-BIL is adjuvant-induced arthritis group administrated BIL (200 mg/kg of body weight daily i.p.).

CO	**N**	
CRP (μg/ml)	5	548.53±19.20
Ceruloplasmin (mg/l)	5	283.70±7.69
AIA	**N**	
CRP (μg/ml)	6	792.55±21.30**
Ceruloplasmin (mg/l)	6	633.29±7.21***
AIA-BIL	**N**	
CRP (μg/ml)	6	543.05±16.55++
Ceruloplasmin (mg/l)	6	509.00±8.97++

Data are expressed as mean ± SEM, 5-6 rats in each group. **, *** statistically significant to CO ($p<0.01$, $p<0.001$); ++ statistically significant to AIA ($p<0.01$).

In the AIA-BIL group compared to the AIA group, we observed a significant decrease of RBC, HGB and HCT at the end of the experiment (Table 3). Interestingly, on day 28, platelets (PLT) were significantly increased in the AIA-BIL group in comparison to AIA (+++ $p<0.001$, Table 3). For AIA in comparison to the CO group this applied on all days monitored (* $p<0.05$, *** $p<0.001$, Table 3).

Discussion

Only few *in vivo* studies have been reported in the past to document modulatory effects of unconjugated BIL on the immune system (Liu *et al.* 2008, Ollinger *et al.* 2007, Wang *et al.* 2004, Kato *et al.* 2003, Khan and Poduval 2011, Kirkby and Adin 2006). As far as we know, this study is the first to record hematologic changes during administration of high doses of unconjugated BIL that generated a clinically relevant concentration in the organism during 14 days.

Numerous earlier reports proved that unconjugated BIL acted as an anti-inflammatory agent (Fischman *et al.* 2010, Vitek 2005). Khan and Poduval

Table 3. Hematological values in time profile. Healthy animals are described as CO, AIA is untreated adjuvant-induced arthritis group and AIA-BIL is adjuvant-induced arthritis group administrated BIL (200 mg/kg of body weight daily i.p.).

CO				
	N	**Day 14**	**Day 21**	**Day 28**
RBC (10^6/μl)	7	0.84±0.02	0.96±0.01	1.00±0.01
HGB (g/dl)	7	1.37±0.02	1.63±0.02	1.66±0.01
HCT (%)	7	4.03±0.07	4.47±0.05	4.76±0.04
PLT (10^3/μl)	7	66.28±2.16	58.43±0.03	63.14±0.001
AIA				
	N	**Day 14**	**Day 21**	**Day 28**
RBC (10^6/μl)	7	0.86±0.02	0.96±0.01	1.05±0.01
HGB (g/dl)	7	1.38±0.01	1.51±0.02	1.60±0.02
HCT (%)	7	3.89±0.03	4.23±0.05	4.50±0.05
PLT (10^3/μl)	7	83.38±1.47*	104.63±0.02***	97±0.001***
AIA-BIL				
	N	**Day 14**	**Day 21**	**Day 28**
RBC (10^6/μl)	7	0.84±0.07	0.91±0.02	0.92±0.04++
HGB (g/dl)	7	1.31±0.02	1.40±0.02	1.42±0.02+
HCT (%)	7	3.68±0.04	3.89±0.04+	3.92±0.06+
PLT (10^3/μl)	7	80.13±1.59	118.29±0.02	130±0.001+++

Data are expressed as mean ± SEM, 7 rats in each group. *, *** statistically significant to CO ($p<0.05$, $p<0.001$); +, ++, +++ statistically significant to AIA ($p<0.05$, $p<0.01$, $p<0.001$).

(2011) studied levels of inflammatory cytokines from macrophages and the toxicity of unconjugated BIL on T cells, B cells and macrophages. Their results suggest that BIL is toxic to cells involved in the adaptive and innate immune system. BIL, at a clinically-relevant concentration of 25 μmol/l, significantly compromised the immune function of B cells and macrophages and induced death in immune cells. The inhibitory effect of BIL on B cell proliferation after lipopolysaccharide stimulation was a result of proliferative arrest in lymphocytes or induction of necrosis and apoptosis in mature immune cells. Although these findings were conducted *in vitro* on cell cultures, BIL administration to mice led to a significant reduction in spleen weight, spleen index, and viability of spleen and bone marrow cells. In our model for multisystem autoimmune disease, we proved a significant decrease of WBC and lymphocyte count that resulted in decreased inflammatory infiltrate in hind paw joints of rats suffering from adjuvant induced arthritis (AIA). Further a significant reduction of thymus and spleen weight was observed. The weight of the spleen in the AIA group was significantly increased, a finding which can be observed also in patients with rheumatoid arthritis (RA). On the other hand, in AIA-BIL the weight was significantly decreased, which correlates with findings of Khan and Poduval (2011) and could be described as a therapeutic effect of unconjugated BIL supplementation.

Our results show a significant decrease of thymus weight in both the AIA and AIA-BIL group. As we proposed earlier, this decrease might be related to an enhanced outflow of mature T cells to peripheral blood (Feketeova *et al.* 2012). Our statement is supported by the results of a significantly increased lymphocyte count in AIA. In AIA-BIL a greater thymus weight decrease was observed, although the number of lymphocytes was equal to CO. This might be caused by apoptosis of T cells. Liu *et al.* (2008) described impacts on T cells in experimental autoimmune encephalomyelitis. In their experiment, BIL significantly inhibited Ag-specific and polyclonal T cell responses, while other similar antioxidants completely lacked this effect. BIL suppressed $CD4^+$ T cell responses at multiple steps. High levels of BIL could induce apoptosis in reactive $CD4^+$ T cells. Bilirubin at non-apoptotic concentrations suppressed $CD4^+$ T cell reactivity. When unconjugated BIL was applied *in vivo* as treatment for experimental autoimmune encephalomyelitis in mice, this autoimmune disease was effectively suppressed (Liu *et al.* 2008). Additionally, a significant decrease in RBC and HBG was observed. These findings are very interesting as they demonstrate the diverse effects of hyperbilirubinemia, possibly affecting not only myelopoiesis.

Our findings of a decrease in WBC, RBC and HGB levels are supported by previous research (Khan and Poduval 2011). On the other hand, we are not able to explain the higher levels of PLT. So far there are no other studies that would elucidate this result. Some papers have been published that discuss the functionality of platelets after exposure to BIL, but none described changes in their count. The uniqueness of our study is the length of BIL administration and the timely capturing of values of the parameters investigated. The study by Khan and Poduval (2011) involved only one dose of BIL and the experiment was terminated after 24 h. Liu *et al.* (2008) performed a longer experiment in which BIL was administered to mice with experimental autoimmune encephalomyelitis for 15 days. Clinical outcomes of the experiment are known but hematological evaluations were not published.

The lifespan of erythrocytes in Lewis rats is approximately 60 days, which could be the reason for a postponed decrease in RBC rather than in WBC. The significant decrease of RBC in AIA-BIL is a very interesting finding. The toxicity of BIL to erythrocytes has been widely discussed. The work of Brito *et al.* (2000), based on observation of hyperbilirubinemic *vs* normobilirubinemic neonates, concluded that increased lipid fluidity and high BIL concentrations promoted membrane BIL translocation and toxicity. Contrary to this conclusion, McDonagh (2007) argued that Gunn rats and patients with severe Crigler-Najjar syndrome had lifelong unconjugated hyperbilirubinemia and did not suffer from any abnormal hemolysis or from intravenous injection of unconjugated BIL, which was used extensively as a liver function test. BIL had been injected into infants and adults in many experimental studies without notable hemolysis. The reason for the hypothesis that BIL might induce erythrocyte hemolysis is based mainly on studies of the cytotoxicity of BIL *in vitro* and not *in vivo,* as reported by Kadl *et al.* (2007).

We suggest that the significant decrease of RBC might be due to suppression of production in bone marrow. Unfortunately, bone marrow cells and their viability were not examined in our study. Nevertheless, this conclusion can be supported by the significant decrease in HGB. As a sequitur, the decreased level of HGB may suggest less oxygen carried by RBC to the cells of other tissues. Splenic macrophages contribute

significantly to the degradation of HGB to unconjugated BIL. These results suggest that increased HGB metabolism in the spleen could contribute to the host immunosuppression as reported by Khan and Poduval (2011) and could also possibly affect the number of PLT.

CRP was examined among the first inflammatory markers for determining anti-inflammatory properties of BIL, as reported by many authors; our results correlate with these findings (Khan and Poduval 2011, Ollinger *et al.* 2007, Kadl *et al.* 2007). This parameter level was lowered by BIL supplementation significantly to the level of healthy controls. Similarly, the ceruloplasmin level was significantly lower in AIA-BIL compared to AIA, but it was higher compared to CO. This is interesting as we expected for ceruloplasmin an outcome closer to CRP, because ceruloplasmin is produced in the liver, as is CRP, and it belongs to the proteins produced during the acute phase of the inflammatory process. Different outcomes for these two proteins might be caused by different activation processes that elevate their levels or by a different half-life of the two molecules.

There are only few studies in the literature about decreased levels of ceruloplasmin in subjects with higher levels of plasma BIL (Corchia *et al.* 1994, Murtaza 2009). There is no indication of further investigation of these observational findings.

From traditional Chinese medicine we know that BIL is recognized as a useful medication. Bilirubin in the form of dried powder from cow gallstones is called "Niu Huang" and is indicated in acute inflammation (Kadl *et al.* 2007). As demonstrated in our results, the effects of BIL are potent and they do have an impact on the specific course of the disease we investigated. The results of clinical improvement in the experimental rats and their quick recovery from AIA are very significant.

Liu *et al.* (2008) studied experimental autoimmune encephalomyelitis and found that treatment with BIL suppressed effectively the disease. In contrast, depletion of endogenous BIL dramatically exacerbated the disease. In summary, their results identified BIL as an important immunomodulator that may protect mammals against autoimmune diseases, indicating its potential in the treatment of multiple sclerosis and other immune disorders.

Other studies also suggest a promising future for this somewhat forgotten agent. In animal experiments it was shown that a single bolus injection of BIL improved the clinical outcome or inhibited tumor cell growth (Ollinger and Kogler 2007). It was implied that it might be helpful to induce the so-called "iatrogenic Gilbert syndrome" as prevention for various diseases (McCarty 2007).

In conclusion, our study proved the anti-inflammatory as well as immunomodulatory properties of BIL. After its supplementation in the animal model of adjuvant arthritis, clinical signs – edemas of hind paws – were significantly decreased, resulting in almost no inflammatory infiltrate of granulocytes in histology of hind paw joints. Blood screening in time profile showed decreased RBC, HGB, WBC and lymphocyte count. PLT were rising. Markers of inflammation (CRP and ceruloplasmin) were decreased and so was the organ weight of the thymus and spleen after BIL supplementation. From the available literature it is obvious that the mechanism of BIL effects lies in leading immune cells into apoptosis. This mechanism might be useful in further application of BIL in additional experiments.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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